INTERACTION OF RIFAMYCIN WITH BACTERIAL RNA POLYMERASE

By Walter Wehrli, Fritz Knüsel, Karl Schmid, and Matthys Staehelin

RESEARCH LABORATORIES, PHARMACEUTICAL DEPARTMENT, CIBA LIMITED, BASEL, SWITZERLAND

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The rifamycins specifically inhibit DNA-dependent RNA polymerase (nucleosidetriphosphate: RNA nucleotidyl transferase, DNA-dependent. E.C.2.7.7.6) of bacterial but not of mammalian origin.[1, 2] Mizuno *et al.*[3, 4] have shown that streptovaricin, a substance chemically related to the rifamycins, also specifically inhibits DNA-dependent RNA polymerase from *E. coli* but not from Ehrlich ascites cells. These authors demonstrated that DNA, CTP, Mg^{++}, or Mn^{++}, do not interact with the antibiotic. We have previously reported (*a*) that inhibition by the rifamycins is dependent on the amount of enzyme present,[2] (*b*) that the sensitivity of bacterial and mammalian cells is strikingly different,[2] and (*c*) that RNA polymerase isolated from bacteria resistant to rifamycin is not affected by the antibiotic.[5] All these findings suggest the occurrence of a direct interaction between enzyme and antibiotic.

Using a ^{14}C-labeled rifamycin derivative, we have now obtained evidence that RNA polymerase forms a very stable complex with the antibiotic with simultaneous loss of activity. Moreover, RNA polymerase from rifamycin-resistant mutants of *E. coli* does not form a complex with the antibiotic and is not inhibited.

Materials and Methods.—CTP-^{3}H was purchased from Schwarz BioResearch (Orangeburg, N.Y.), unlabeled nucleotide triphosphates and spermidine hydrochloride from Calbiochem (Lucerne, Switzerland), and thymus DNA from Fluka (Buchs, Switzerland). The rifamycin derivatives used in this work were rifampicin,[6] rifamycin B,[7] and rifamycin SV.[8] ^{14}C-labeled rifampicin (spec. act. 2.5 $\mu c/\mu mole$) was prepared as previously described.[9] All other chemicals were of commercially available analytical grade.

RNA polymerase was prepared from logarithmically growing *E. coli* ETH 2018, as described in a previous paper.[2] Under standard conditions, 1 mg of protein incorporated 150–300 mμmoles of ^{3}H-CMP. Mutant *E. coli* cells resistant to rifampicin were selected and grown as described previously,[5] and the RNA polymerase was isolated in the same way as the enzyme from sensitive cells. Under standard conditions, 1 mg of protein incorporated 50–100 mμmoles of ^{3}H-CMP.

Polymerase assay: The usual reaction mixture contained 20 μmoles of Tris-HCl (pH 7.9); 2.5 μmoles of β-mercaptoethanol; 2.0 μmoles of $MgCl_2$; 0.4 μmole of ATP; 0.2 μmole each of GTP and UTP; 0.05 μmole of ^{3}H-CTP (spec. act. 10 $\mu c/\mu mole$); 50 μg of DNA (calf thymus); 20 μmoles of NH_4Cl, 2 μmoles of spermidine-HCl, 20 μg of protein in a final volume of 0.25 ml, and was incubated for 20 min at 37°. The reaction was stopped by a cooling in ice and the immediate addition of 0.5 ml of 2 *N* HCl. The precipitate was collected on a glass filter disk (Whatman GF/C, 2.4-cm diameter), washed with dilute HCl, dried and counted on a Packard Tri-Carb liquid scintillation counter. A scintillation solution of 6 gm Butyl PBD (CIBA) in 1 liter of toluene was used.

Isolation of rifampicin-enzyme complex by gel filtration: From either sensitive or resistant cells, 0.25 ml of partially purified enzyme (0.60 mg protein) was mixed with 1.75 μg (2.13 mμmoles) of ^{14}C-rifampicin in 0.01 ml of H_2O and incubated for 10 min at 37°. After cooling in ice, the mixture was chromatographed on a 2 × 14-cm column of Sephadex G75 at 4° in order to separate bound from free rifampicin. The elution buffer contained 0.01 *M* Tris-HCl (pH 7.9), 0.01 *M* $MgCl_2$, 0.1 mM EDTA, 0.1 *M* $(NH_4)_2SO_4$, 0.01 *M*

2-mercaptoethanol, and 1 mg/ml of bovine serum albumin (BSA); 0.9 ml of each 1-ml fraction was directly dried on a glass filter disk in a polyethylene counting vial at 120° and counted, and 0.1 ml of each 1-ml fraction was analyzed for polymerase activity. In the absence of rifampicin the recovery of polymerase activity was 30–40%.

Sucrose gradients: Of the partially purified sensitive or resistant enzyme (0.24 and 0.28 mg of protein, respectively), 0.1 ml was mixed with 0.7 μg of ^{14}C-rifampicin in 0.01 ml H_2O and incubated for 10 min at 37°. After cooling, the mixture was layered on a 5-ml linear gradient of 20–5% sucrose in a solution containing 0.05 *M* KCl, 0.01 *M* Tris-HCl (pH 7.9), 5 mM 2-mercaptoethanol, and 0.5 mM EDTA.[10] After centrifugation for 3 hr at 65,000 rpm at 0–2°, the tubes were punctured and 0.4-ml fractions were collected. The rifampicin content was determined by mixing each fraction with 20 ml methanol–toluene (1:1) containing scintillator and counting on a Packard liquid scintillation counter. To determine the RNA-polymerase activity in the absence of rifampicin, gradients without antibiotic were run under otherwise identical conditions; 0.01 ml of each fraction was assayed for enzyme activity under standard conditions. The yield of polymerase activity was 35–45%.

Results.—Polymerase from rifampicin-sensitive cells: Chromatography of RNA polymerase on Sephadex separates the enzyme from low molecular substances. Although BSA and mercaptoethanol were added to the elution buffer as stabilizers, the recovery of enzyme activity was only 30–40 per cent. To determine whether rifampicin would form a complex with the enzyme, a mixture of rifampicin and RNA polymerase was incubated and chromatographed on Sephadex G 75 as described under *Materials and Methods.* Figure 1 shows that ^{14}C-rifampicin is eluted in two well-separated peaks. The first peak appears in

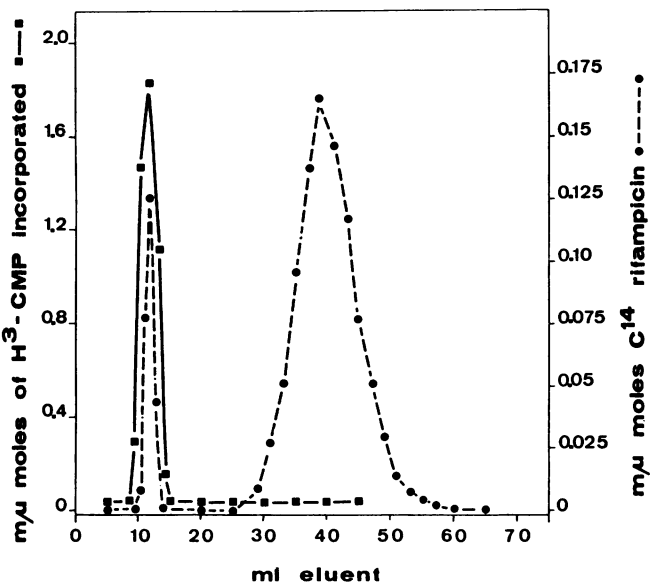

FIG. 1.—Determination of the binding of ^{14}C-rifampicin to *E. coli* RNA polymerase by chromatography on Sephadex G 75.

■——■, RNA-polymerase activity (mμmoles of ^{3}H-CMP incorporated per 0.1 ml of each 1-ml fraction). The recovery of polymerase activity was 30–40%.

●- - -●, Rifampicin (mμmoles per 1-ml fraction). Note that in the peak of free rifampicin only, each second fraction is shown.

the same position as RNA polymerase in the absence of rifampicin. The second peak corresponds to free rifampicin. Control experiments with BSA and DNA showed only the small-molecular-weight peak; no radioactivity was bound to the macromolecular fraction.

To confirm that RNA polymerase itself (and not a contaminating high molecular substance) forms a complex with rifampicin, the sedimentation pattern of the protein-bound rifampicin was compared with that of RNA polymerase on a sucrose density gradient. As shown in Figure 2, the radioactivity is split into two peaks, one of them having the same S value as RNA polymerase.

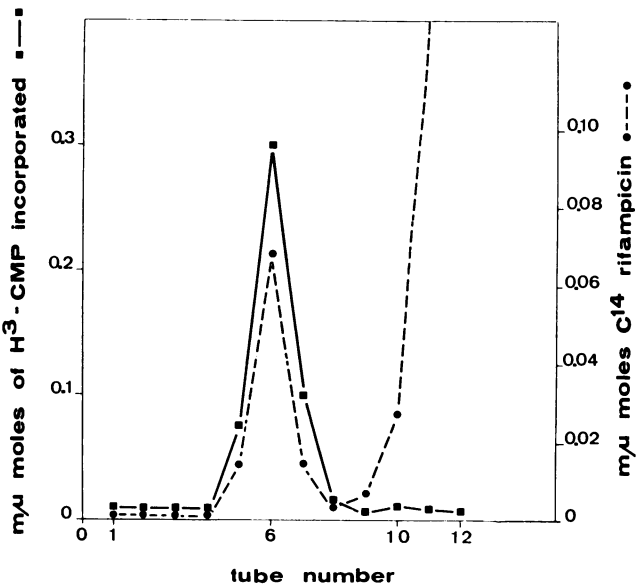

FIG. 2.—Determination of the binding of ^{14}C-rifampicin to *E. coli* RNA polymerase on a sucrose density gradient.

■——■, RNA-polymerase activity (mμmoles of ^{3}H-CMP incorporated per 0.01 ml of each 0.4-ml fraction). The recovery of polymerase activity was 35–45%.

●- - - -●, Rifampicin (mμmoles per 0.4-ml fraction).

Polymerase from rifampicin-resistant cells: RNA polymerase from rifampicin-resistant *E. coli* did not form a complex with the antibiotic, as is apparent from the elution pattern on Sephadex (Fig. 3). The same result was obtained on a sucrose gradient (Fig. 4). The resistant polymerase sediments like the normal enzyme, but does not bind any rifampicin.

Properties of the rifampicin-enzyme complex: With a four- to tenfold excess of rifampicin the complex is readily formed on being incubated for ten minutes at 37°. Larger amounts of antibiotic and longer incubation times do not significantly enhance complex formation. Incubation even for five minutes at 0° yields about half as much complex as does incubation for ten minutes at 37°. Figure 5, in which enzyme inhibition and rifampicin binding are plotted as a function of the rifampicin concentration, shows that formation of the rifampicin-

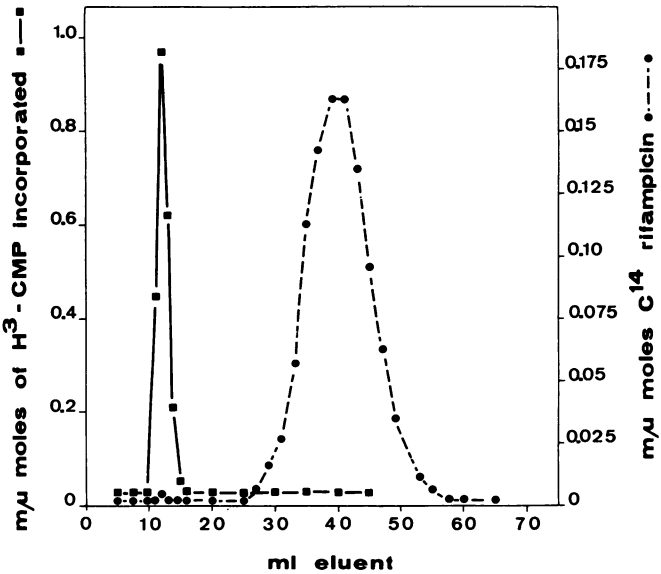

FIG. 3.—Determination of the binding of ^{14}C-rifampicin to RNA polymerase from rifampicin-resistant cells by chromatography on Sephadex G 75. Conditions as in Fig. 1. ■——■, RNA-polymerase activity; ●- - -●, rifampicin.

enzyme complex is the reaction responsible for inactivation of the enzyme. As expected, an increase in complex formation goes hand in hand with an increased inhibition of polymerase activity.

The fact that rifampicin remains bound to the enzyme after chromatography on Sephadex, or after density gradient centrifugation, suggests that a rather stable complex is formed. Dissociation seems to be very slow, since rechromatography of the isolated complex on Sephadex caused only minor decomposition. That rifampicin is indeed strongly bound to the enzyme could be demonstrated by incubating the complex formed with unlabeled rifampicin in the presence of labeled rifampicin, or vice versa (Table 1). When RNA polymerase was incubated first with labeled rifampicin for ten minutes at 37°, and then with additional unlabeled antibiotic, less than 5 per cent radioactivity was lost. On the other hand, only traces of radioactive rifampicin were bound when the enzyme was first incubated with unlabeled and then with labeled antibiotic. With this technique it was found (Table 1, expts. 5 and 6) that other rifamycin derivatives such as the naturally occurring rifamycin B[7] and rifamycin SV,[8] which are also inhibitors of RNA polymerase,[1, 2] form a similar complex with the enzyme (Table 1).

Since the complex of polymerase with DNA is not formed at salt concentrations over 0.3 *M*,[11] the interaction of the enzyme with rifampicin was studied at various salt concentrations and found not to be significantly affected even by 1.0 *M* salt; 0.1 ml of enzyme (0.24 mg of protein) and 0.1 ml 2 *M* KCl were mixed with 0.7 μg rifampicin in 0.01 ml H_2O and incubated for ten minutes at 37°. After cooling, the mixture was chromatographed on Sephadex G 75 and the

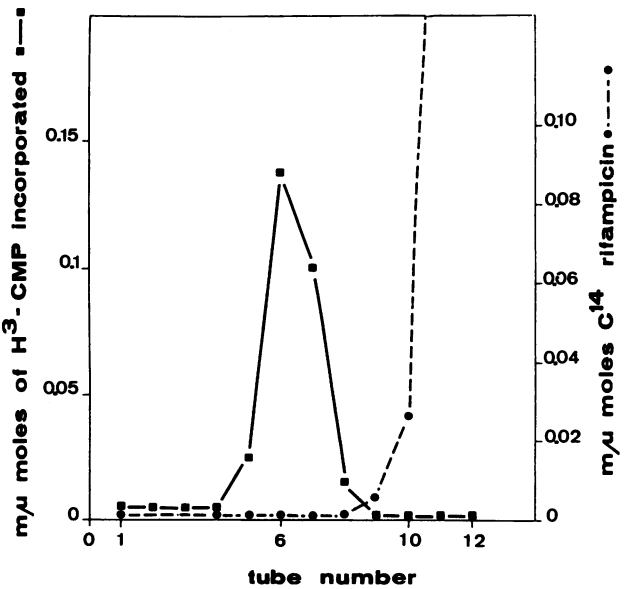

FIG. 4.—Determination of the binding of ^{14}C-rifampicin to RNA polymerase from rifampicin-resistant cells on a sucrose gradient. Conditions as in Fig. 2. ■——■, RNA-polymerase activity; ●- - -●, rifampicin.

TABLE 1. *Stability of the rifampicin-enzyme complex.*

Expt. no.*	Antibiotic present during first incubation	Antibiotic added for second incubation	^{14}C-rifampicin (μmoles) bound to RNA polymerase
1	^{14}C-rifampicin	None	0.25
2	^{14}C-rifampicin	Unlabeled rifampicin	0.24
3	None	^{14}C-rifampicin	0.25
4	Unlabeled rifampicin	^{14}C-rifampicin	0.01
5	Rifamycin B	^{14}C-rifampicin	0.01
6	Rifamycin SV	^{14}C-rifampicin	0.01

* (1) 0.25 ml of the partially purified enzyme (0.60 mg of protein) was mixed with 1.75 μg of ^{14}C-rifampicin in 0.01 ml of water and incubated for 10 min at 37°.

(2) After incubation of the same amount of enzyme and ^{14}C-rifampicin as in (1), 1.75 μg of unlabeled rifampicin in 0.01 ml of water was added and the mixture incubated for another 10 min at 37°.

(3) After incubation of 0.25 ml of enzyme for 10 min at 37° with 0.01 ml of water, 1.75 μg of ^{14}C-rifampicin in 0.01 ml of water was added and the mixture incubated for another 10 min at 37°.

(4) Same amounts and conditions as in (2), but the enzyme was first incubated with unlabeled and then with ^{14}C-labeled rifampicin.

(5) As in (4), but instead of unlabeled rifampicin, rifamycin B was used.

(6) As in (4), but instead of unlabeled rifampicin, rifamycin SV was used.

In all cases the complex was isolated on Sephadex G75 columns and determined as described under *Materials and Methods*.

bound rifampicin determined as described under *Materials and Methods*, except that the elution buffer also contained in addition 1 *M* KCl. The yield of enzyme-antibiotic complex obtained under these conditions was at least 85 per cent of that formed in the absence of KCl. On the other hand, treatment of 0.1 ml of enzyme with 0.1 ml 1% Na-dodecylsulfate destroyed its ability to form a complex with rifampicin.

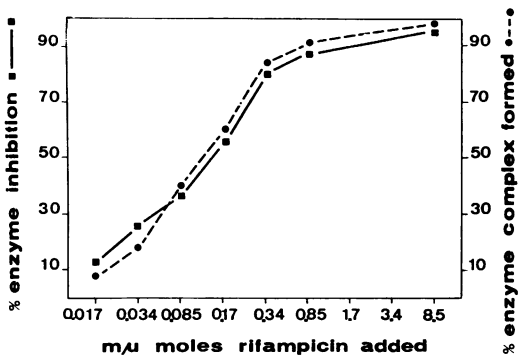

FIG. 5.—Relationship between the extent of complex formation and the inhibition of RNA polymerase. 0.1 ml of partially purified normal *E. coli* RNA polymerase (0.24 mg protein) was mixed with the indicated amounts of rifampicin and incubated for 10 min at 37°. The complex was isolated on Sephadex G 75 columns and determined as described under *Materials and Methods*. The amount of complex formed after incubation for 30 min at 37°, using 17 mμmoles of rifampicin, was taken as 100%. 0.1 ml of each fraction was assayed for remaining polymerase activity.

Discussion and Summary.—The results reported in this paper indicate that rifampicin and other rifamycin derivatives form a very stable complex with RNA polymerase. Since no such complex is formed with the enzyme derived from rifampicin-resistant cells, it would appear that formation of the complex is responsible for inactivation of the enzyme. This is further borne out by the observation that inhibition of the enzyme is proportional to the amount of complex formed. No definite values can as yet be given for the stoichiometry of the rifampicin-enzyme complex. Assuming a purity of 5–20 per cent for our polymerase preparation, and a molecular weight of 720,000 daltons,[12] it can be calculated from the data of Figures 1 and 2 that 1.5–6 molecules of rifampicin bind to 1 molecule of enzyme.

The ready formation of the complex, its stability in a rifampicin-free medium, and the finding that there is but a slow exchange of bound with free antibiotic, all indicate that the equilibrium is strongly in favor of the complex. Since the complex is formed in the presence of 1.0 *M* KCl, under conditions in which the enzyme fails to bind its DNA template[12] so that RNA synthesis is blocked at the first step,[13, 14] it appears that the binding sites for rifampicin and DNA are different. The same conclusion may be drawn from the finding[15] that formation of the DNA-enzyme complex has no influence on the inhibitory effect of rifampicin.

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Abbreviations: CMP, cytidine 5′-phosphate; ATP, adenosine 5′-triphosphate; CTP, cytidine 5′-triphosphate; GTP, guanosine 5′-triphosphate; UTP, uridine triphosphate; EDTA, ethylenediaminetetraacetate; Tris-HCl, tris(hydroxymethyl)aminomethane-HCl.

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